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HERBIVORE AVOIDANCE OF DIGITALIS EXTRACTS IS NOT MEDIATED BY CARDIAC GLYCOSIDES

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Abstract—This study was conducted to determine whether avoidance of digitalis (*Digitalis purpurea*) by mountain beaver (*Aplodontia rufa*) is induced by toxic cardiac glycosides. High-performance liquid chromatography and behavioral assays were used to relate animal responses with the presence of common cardiac glycosides in several digitalis extracts. Statistical analyses of multiple-choice tests showed no correlation between cardiac glycoside content and mountain beaver avoidance of apple cubes treated with digitalis extracts. Therefore, we concluded that known toxic cardiac glycosides were not responsible for chemosensory cues that inhibited intake of food treated with digitalis extracts. These results suggest that digitalis is a source of an effective nontoxic herbivore repellent.

Key Words—*Aplodontia rufa*, avoidance, cardiac glycoside, digitalis, *Digitalis purpurea*, herbivore, mountain beaver, repellent

INTRODUCTION

Human and wildlife interactions are becoming more frequent, often with negative effects on both humans and wildlife. Current preventive measures such as trapping and lethal control are often not feasible or inappropriate in many management situations. Nonlethal repellents may provide viable alternatives.

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Plants that are naturally avoided may be a source of aversive agents to inhibit browsing (Cardellina, 1988). For example, digitalis (*Digitalis purpurea*) plants are rarely harvested by mountain beaver (*Aplodontia rufa*), and prior studies indicate that even a preferred food is avoided when treated with water extracts of digitalis (Nolte et al., 1995). Unfortunately, toxins may render digitalis undesirable as a source of nonlethal aversive agents. The aversive cues, however, are not necessarily the toxic cardiac glycosides. Gustatory or olfactory cues of a food, and the specific compounds that cause aversive feedback, need not be synonymous (Provenza and Balph, 1990). We conducted a series of chemical and behavioral assays to determine whether the toxic glycosides common in digitalis are the aversive cues avoided by mountain beaver.

METHODS AND MATERIALS

Chemicals. High-performance liquid chromatography (HPLC) grade methanol, acetonitrile, and hexane were obtained from Fisher Scientific (Fair Lawn, New Jersey), and HPLC grade chloroform was obtained from Baxter (McGaw Park, Illinois). Water was either HPLC grade (Baxter) or purified in-house using the Milli-Q+ purification system (Millipore, Molsheim, France). Digitoxin and gitoxin were obtained from Sigma (St. Louis, Missouri).

Stimuli. Digitalis was collected in the vicinity where test animals were subsequently trapped. Samples were frozen and lyophilized for 48 hr. The lyophilized material was ground through a 20-mesh Wiley mill, mixed uniformly, and stored at 4°C.

Subjects. Experimentally naive (not previously tested) adult mountain beavers were trapped in the Capital State Forest, Grays Harbor County, approximately 30 km from Olympia, Washington. Animals were penned individually in outdoor pens (3 × 3 m) and given free access to pelleted feed (X-Cell Feed Company, Tacoma, Washington) and water throughout the trials. All animals were given a minimum of four days to adjust to captivity and the test regime prior to the onset of trials.

Extract Preparation. To derive a series of liquid–liquid extracts, two hot water digitalis extracts were prepared identically by placing 60 g of ground digitalis in 1.5 liters of water maintained at 85°C. The extraction was performed for 4 hr with periodic stirring followed by filtration through Whatman 114 coarse filter paper (Whatman International Ltd., Maidstone, Kent, U.K.). The liquid–liquid extracts were subsequently prepared by sequentially "washing" one of the filtered hot water digitalis extracts with 1.2 liters of hexane and 1.2 liters chloroform in a separatory funnel by manual shaking for 20 min. The hexane (L-L:H1) and chloroform (L-L:C2) phases, as well as the resultant filtrate (RF:A) were collected. This process was repeated with the other hot water digitalis extract but the order of the hexane and chloroform washes was switched. Again, the chloroform (L-L: C1) and hexane (L-L: H2) phases were collected as was the resultant filtrate (RF: B).

Room temperature liquid–solid extracts were prepared by combining 60 g of ground digitalis with methanol (L-S: M), hexane (L-S: H), chloroform (L-S: C), or water (L-S: W) and shaking on a horizontal mechanical shaker for 30 min prior to filtration. Approximately 1.25 liters of filtrate was collected from each of the extraction processes.

Digitalis extracts were applied to apple cubes (1 cm^3) by submerging the cubes in the extracts for 1 hr immediately prior to tests. Apple cubes were similarly treated with the respective solvents for trials to determine whether mountain beaver were responding to cues emitted from digitalis or merely avoiding the solvents. Peeled apples were selected as the test food because they are readily ingested by mountain beavers.

Behavioral Assays. A series of multiple-choice tests were used to determine mountain beaver responses to extracts derived from digitalis. First, mountain beaver were given a choice of apple cubes treated with the liquid–liquid extracts (L-L: H1, L-L: H2, L-L: C1, L-L: C2) and the resultant filtrates (RF: A, RF: B) along with a water control. Response of mountain beavers to the liquid–solid extracts (L-S: H, L-S: C, L-S: M, L-S: W), one of the resultant filtrates (RF: B), and a water control was assessed in the second test. A third behavioral assay was conducted with the solvents (methanol, hexane, chloroform, and a water control) used to prepare the extracts.

During each of the tests, mountain beaver (N = 10) were presented with 20 untreated apple cubes placed in weigh-boats spaced at even intervals along the perimeter of their pens for four days of pretreatment. The number of apple cubes in each weigh-boat and within the immediate area (<30 cm) was assessed after 24 hr. At that time, all remaining cubes were removed and another 20 fresh cubes were placed in the weigh-boats. A four-day treatment period immediately followed pretreatment. Treatment procedures were identical to those described for pretreatment except apples cubes were treated with the extracts as described above. Three tests were conducted sequentially as described above, and all treatments (extracts) within a test were offered simultaneously. Treatment locations were randomly selected each day.

Chemical Assays. All extracts were analyzed for digitoxin and gitoxin with a Hewlett Packard 1090M HPLC equipped with a diode array detector (Hewlett Packard Co., Palo Alto, California). Ultraviolet (UV) detection of the analytes was achieved at 220 nm. The analytical column was a 250 × 4.6 mm Keystone Octyl-H with a 10 × 4-mm guard of the same stationary phase (Keystone Scientific, Inc., Bellefonte, Pennsylvania). The mobile phase consisted of 68% water and 32% acetonitrile for 10 min, followed by a 15-min linear gradient of increasing acetonitrile concentration until a composition of 60% water and 40%

acetonitrile was achieved. For chloroform extracts only, the mobile phase consisted of a linear gradient from 100% water to 50% methanol/50% acetonitrile over 65 min. The flow rate was 1.0 ml/min and the injection volume was 10 μl.

Standards of digitoxin and gitoxin, prepared in methanol, were used for identification and quantitation.

Statistical Analyses. The data for each behavioral assay were assessed separately in single factor analyses of variance (ANOVA). A randomized block design was used where mountain beaver were blocks and food with the respective extracts were treatments. Tukey tests (Winer, 1971) were used to isolate significant differences among means subsequent to the omnibus procedures ($P < 0.05$).

RESULTS

Mountain beaver ($N = 9$) responses varied among treatments in the first behavioral assay ($P < 0.0001$) (Figure 1). One animal was not included in the

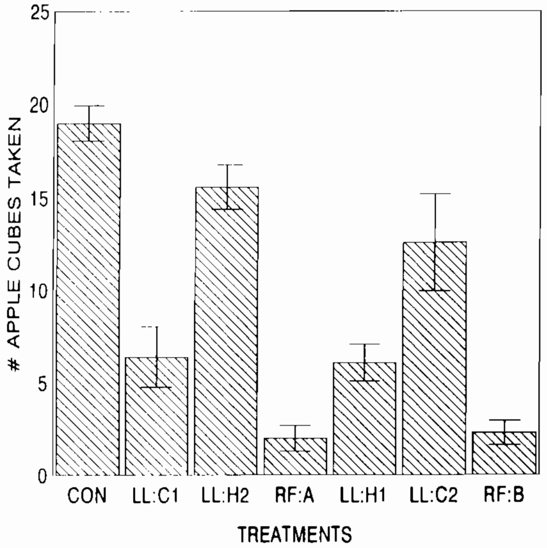

FIG. 1. Number of apple cubes take by moutain beaver during a multiple-choice test when the test food (20 apple cubes) was treated with one of six digitalis extracts prepared by a two-step liquid–liquid extraction or untreated (CON). The resultant filtrate (RF : A) was first collected after hexane (LL : H1) was followed by chloroform (LL : C2). Next the resultant filtrate (RF : B) was collected after chloroform (LL : C1) was followed by hexane (LL : H2).

analysis because it failed to ingest any treatment or control cubes throughout the assay. Mountain beaver took fewer ($P < 0.05$) apple cubes treated with either of the resultant filtrates (RF:A, RF:B) or the initial phase L-L extracts (L-L:H1, L-L:C1) than they did control cubes or cubes treated with the second phase liquid–liquid extracts (L-L:H2, L-L:C2). Mountain beaver responses were similar ($P > 0.05$) to L-L:H2 and L-L:C2 and to L-L:H2 extract and control cubes, but they took fewer ($P < 0.05$) cubes treated with the L-L:C2 extract than control cubes. Subsequently, we rated the mountain beavers' relative avoidance of each extract as: high, greater than control; low, similar to control; or moderate, greater than control but less than extracts rated as high (Table 1).

Responses of mountain beavers also varied with treatments in the second behavioral assay ($P < 0.0001$) (Figure 2). Animals ingested similar numbers of cubes treated with the hexane (L-S:H) or chloroform (L-L:C) extracts as they did control cubes ($P > 0.05$). Response to the water extracts (L-S:W or RF:B) and the methanol (L-S:M) extract were similar, and all of these extracts were substantially more aversive than the other treatments ($P < 0.05$). Mountain beavers' relative avoidance of these extracts was also rated as described above (Table 1).

None of the solvents used to prepare extracts were aversive. Mountain

TABLE 1. SUMMARY OF MOUNTAIN BEAVER RESPONSES TO TEST EXTRACTS AND ABSENCE OR PRESENCE OF CARDIAC GLYCOSIDES

Extract	Cardiac glycosides	Relative avoidance
Liquid–solid		
L-S:W	present	high
L-S:M	absent	high
L-S:C	absent	low
L-S:H	absent	low
Liquid–liquid		
L-L:C1	present	high
L-L:H2	absent	low
R-F:A	absent	high
L-L:H1	present	high
L-L:C2	present	moderate
R-F:B	absent	high
Solvents		
Water	absent	low
Methanol	absent	low
Chloroform	absent	low
Hexane	absent	low

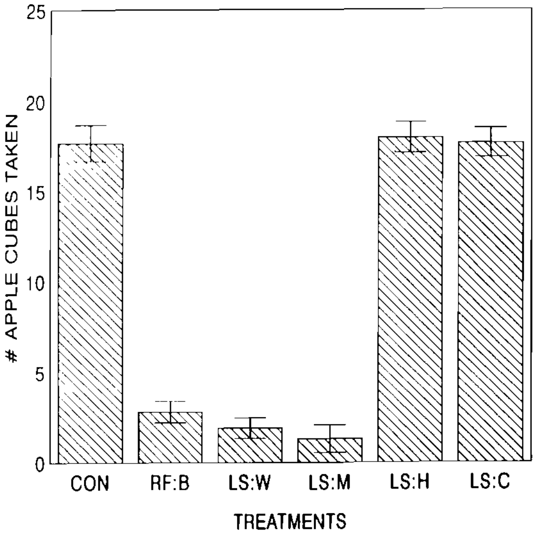

FIG. 2. Number of apple cubes taken by mountain beaver during a multiple-choice test when the test food (20 apple cubes) was treated with one of four liquid–solid digitalis extracts prepared with methane (LS:M), chloroform (LS:C), hexane (LS:H), water LS:W), or treated with the resultant filtrate (RF:B) from a chloroform/hexane liquid–liquid extract, or untreated (CON).

beaver ingested similar numbers of apple cubes in the third assay regardless of treatment ($P > 0.3$). The mean number of the 20 apple cubes treated with methanol, chloroform, hexane, or water taken by mountain beaver was 19.2, 19.7, 17.8, and 18.0, respectively.

Chemical Assays. All extracts were analyzed for digitoxin and gitoxin to determine if the cardiac glycosides were present (Table 1). Digitoxin and gitoxin were detected in the hot water liquid–solid extract prior to being subjected to the liquid–liquid extraction. HPLC analysis also indicated that digitoxin and gitoxin were present in both chloroform liquid–liquid extracts (L-L:C1 and L-L:C2), as well as the initial hexane liquid–liquid extract (L-L:H1) (Figure 3). Cardiac glycosides were not present in the second hexane L-L extract (L-L:H2) or either of the resultant filtrates (RF:A and RF:B) (Figure 4). Although the water liquid–solid extract contained digitoxin and gitoxin, neither of these cardiac glycosides were detected in the other liquid–solid extracts (L-S:M, L-S:H, L-S:C).

The detection limits, defined as the analyte concentration required to produce a chromatographic response equal to three times the chromatographic noise,

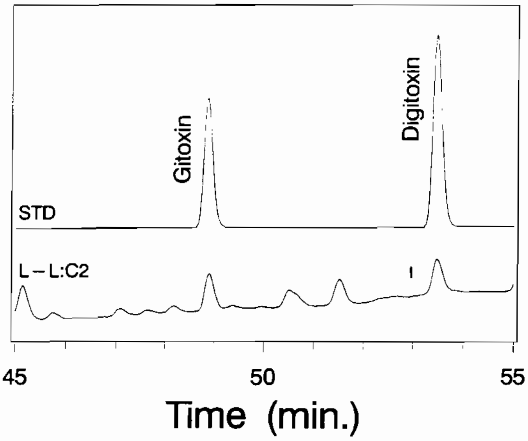

FIG. 3. Chromatograms of an extract (L-L:C2) in which digitoxin and gitoxin were detected and a standard mixture of gitoxin and digitoxin (STD). The y scale of L-L:C2 was amplified by fourfold for clarity. Chromatographic conditions are provided in the text.

were found to be 0.67 μg/ml and 0.48 μg/ml for digitoxin and gitoxin, respectively.

DISCUSSION

Aversive cues emitted by foxglove do not appear to originate from digitoxin or gitoxin. The methanol liquid–solid extract (L-S:M) and both resultant filtrates

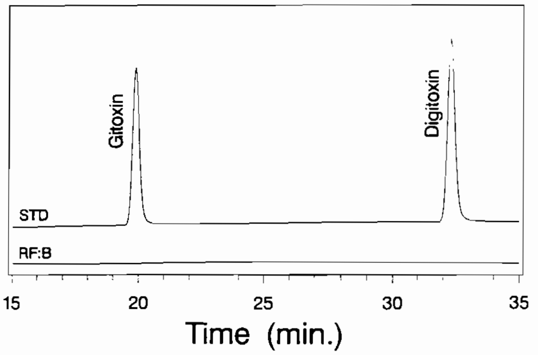

FIG. 4. Chromatograms of a resultant filtrate (RF:B) in which digitoxin and gitoxin were not detected and a standard mixture of digitoxin and gitoxin (STD). Chromatographic conditions are provided in the text.

were avoided by mountain beaver, although digitoxin and gitoxin were absent. In addition, avoidance of one of the liquid–liquid extracts that contained both digitoxin and gitoxin was only moderate.

The absence of cardiac glycosides in extracts was based on chromatographic analysis of the extracts for digitoxin and gitoxin, although other cardiac glycosides are known to be present in digitalis (Fujii et al., 1989). Digitoxin and gitoxin, however, should be good indicators of the cardiac glycosides present in digitalis. Digitoxin and gitoxin are two of the three main secondary glycosides formed from the primary glycosides that are present in living, undamaged digitalis plants. The polarities and solubilities of all the primary and secondary glycosides are similar. Therefore, we used the presence of digitoxin and gitoxin as "markers" for the presence of all possible cardiac glycosides. If neither digitoxin nor gitoxin were detected in an extract, it was assumed that no cardiac glycosides were present in the extract at our detection limit.

The combined assays indicate that the chemical cues avoided by mountain beaver were polar. Extracts prepared with polar solvents were aversive to mountain beaver, while extracts prepared with nonpolar solvents were generally not avoided. For example, in the second behavioral assay, apple cubes treated with the nonpolar hexane (L-S:H) and chloroform (L-S:C) extracts were taken, while cubes treated with the polar water (L-S:W) and methanol (L-S:M) extracts were avoided. Furthermore, the aversive resultant filtrates (RF:A, RF:B) were also derived from polar extracts of digitalis.

Generally, the polar extracts were avoided by mountain beaver on the first day of trials. Repellents that elicit initial avoidance are generally either irritants (e.g., capsaicin) or those that evoked "fear" response (e.g., predator scents) (Mason and Clark, 1992). The initial avoidance in these trials, however, may have reflected a conditioned food aversion. Conditioned food aversions occur when ingestion of a novel food is paired with gastrointestinal distress (Garcia and Koelling, 1966; Garcia, 1989). The mountain beavers in our trials likely had encountered digitalis prior to capture. Since the glycosides in digitalis induce nausea, it is possible that these animals learned to avoid digitalis through pairing the nontoxic cues with the toxins. Gustatory or olfactory cues of a food, and the specific compounds that cause aversive feedback, need not be and probably rarely are synonymous (Provenza and Balph, 1990).

Management Implications. Digitalis extracts may provide an effective source of aversive agents to inhibit herbivore damage in the Pacific Northwest. Nontoxic cues derived from the plant are avoided by mountain beaver. However, it is unclear whether the avoidance reflects a conditioned aversion. Regardless, the abundance of digitalis throughout the Pacific Northwest virtually ensures that if learning is required, animals will recognize its aversive properties through prior "natural" experiences. Further, herbivore familiarity with digitalis may reduce habituation to its aversive cues. Animals that ignore cues normally emitted by the plant would risk exposure to its toxic properties.

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